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Structural determination of the active site of a sweet protein

A ^{1}H NMR investigation of pMNEI

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pMNEI, a single chain sweet protein related to monellin, has been studied by means of ^{1}H NMR at 500 MHz. A partial sequential assignment performed by means of the MCD method allowed the determination of the secondary structure of a large portion of the β-sheet of pMNEI that contains a likely 'sweet finger': the loop connecting the β-strands from residue 59 to residue 78, corresponding to segment 16–35 of the A chain of monellin. The detailed three-dimensional structure of the loop (Tyr^{66}-Ala^{67}-Ser^{68}-Asp^{69}), determined from several interresidue and intraresidue NOEs and subsequent energy minimization, shows that the side chains of Tyr^{66} and Asp^{69} fit our model of the sweet receptor in a manner very similar to that of the side chains of Phe and Asp of aspartame.

Sweet receptor; Monellin; NMR; Molecular mechanics

1. INTRODUCTION

The interaction of sweet molecules with their receptor can be studied in greater detail than most other agonist–receptor interactions, owing to the very large number of active molecules available and to the fact that they belong to classes of widely different chemical constitution [1]. These circumstances have stimulated the development of general models of the receptor active site [2–12].

We have proposed models for sweet [6] and bitter [7] receptors that can discriminate among sweet, bitter and tasteless compounds of very similar chemical constitution but slightly different shape. The main features of our model can be summarised as follows. (i) The active site of the receptor is a shallow, flat cavity with the outer side accessible even during the interaction with the agonist and the inner side defined by the so-called Shallenberger barrier [4] (vide infra, Figs. 2 and 3). (ii) The lower part of the cavity, the shape of which has been identified through several rigid molecular molds [6–8], contains the main electronic features, the most important of these being the AH-B entity; this part is always essential for the binding. (iii) The upper part is hydrophobic and plays an important role in the case of very active tastants. Point (i) is consistent with the fact that there are both synthetic and natural sweet macromolecules, e.g. the two sweet proteins monellin and thaumatin. A further, decisive test of the model would then be the fit of the active site of a sweet protein.

We have undertaken the NMR study of the structure of pMNEI, a single chain monellin obtained through protein engineering methods. Native monellin is a heater dimer protein of molecular weight 11 kDa consisting of subunit A (44 amino acids) and subunit B (50 amino acids) associated by non-bonded interaction. The crystal structure of native monellin [13] indicated that the amino terminal region of subunit A and the carboxyl terminal region of subunit B form a typical antiparallel β-sheet. The amino terminal of subunit A and carboxyl terminal of subunit B were connected by a short linker sequence designed to maintain the topology of the two β-strands in pMNEI. Choice of length and amino acid sequence of the linker was determined on the basis of known crystal structures containing similar parts of β-strands, which were computationally searched in the Protein Data Bank. This single chain monellin is not only as active (sweet) as the native parent molecule but also endowed with enhanced thermal stability.

A likely active site of this very sweet protein is the loop connecting the β-strands from residue 59 to residue 78, corresponding to segment 16–35 of the A chain of monellin [13]. A partial sequential assignment performed by means of the MCD method [14] allowed the determination of the secondary structure of a large por-

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Abbreviations: pMNEI, single chain monellin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; DQF-COSY, double-quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; MCD, main chain directed assignment; MM, molecular mechanics.

tion of the β-sheet of pMNEI, including segment 59–78. The detailed three-dimensional structure of the loop (Tyr[66]-Ala[67]-Ser[68]-Asp[69]), determined from several interresidue and intraresidue NOEs and subsequent energy minimization, shows that the side chains of Tyr[66] and Asp[69] fit our model of the sweet receptor in a manner very similar to that of the side chains of Phe and Asp of aspartame [6].

2. MATERIALS AND METHODS

pMNEI was obtained from *E. coli* strain W3110, transformed by an expression plasmid (pST6311) as described in the paper by Iijima et al. (this journal). The protein is characterised by the following amino acid sequence:

Met[0] Gly[1] Glu Trp Glu Ile Ile Asp Ile Gly Pro Phe Thr Gln Asn Leu Gly Lys Phe Ala Val[20] Asp Glu Glu Asn Lys Ile Gly Gln Tyr Gly Arg Leu Thr Phe Asn Lys Val Ile Arg Pro[40] Cys Met Lys Lys Thr Ile Tyr Glu Asn Glu **Gly Phe** Arg Glu Ile Lys Gly Tyr Glu[60] Tyr Gln Leu Tyr Val Tyr Ala Ser Asp Lys Leu Phe Arg Ala Asp Ile Ser Glu Asp Tyr[80] Lys Thr Arg Gly Arg Lys Leu Leu Arg Phe Asn Gly Pro Val **Pro Pro Pro.**

Residues in bold, including the **Gly Phe** linker between chains A and B, are not present in native monellin.

The protein has a sweetness equivalent to that of native monellin (0.05–0.1 μg/μl threshold concentration) but an enhanced stability, as shown by the fact that it retains sweetness after treatment at 100°C for 20 min at pH 2.3. M.W. is 10.2 kDa (gel filtration/electrophoresis).

All samples for NMR measurements were approximately 3 mM. ^{1}H spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer or at 400 MHz on a Bruker AM-400 spectrometer. The water signal was suppressed by a low-power selective irradiation in the homogated mode. 2D experiments were performed as follows. For phase-sensitive DQF-COSY [15], NOESY [16] and TOCSY [17] experiments 512 FIDs with 128 scans of 2048 points were recorded over 6024 Hz of spectral width at 500 MHz and over 4500 Hz at 400 MHz. Gaussian window functions were applied before transformation in both dimensions. Pure absorption NOESY spectra were obtained with different mixing times with a 10%-random variation of mixing time to cancel scalar correlation effects. Irradiation of the ^1H_2O resonance in 2D experiments was carried out during the relaxation time and, for NOESY, also during the mixing time. Distance constraints were derived from the NOESY spectrum recorded with a 100 ms mixing time, using the method of Esposito and Pastore [18] after correction for spin diffusion according to the method of Majumdar and Hosur [19]. Relative scaling of the constraints was achieved by using two limiting values for typical $d_{\alpha N}$ distances: 2.1 and 2.7 Å, corresponding to ψ values of 120° and 180°, respectively.

Molecular mechanics calculations were performed with the all atom AMBER [20] force field, using a distance depending dielectric constant ($\varepsilon = 10\,r$). The computational procedure can be divided into two steps: (i) an energy minimisation calculation is performed, using a quasi-Newton method, the Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm [21], stopping when the gradient norm is 10^{-3} or less; and (ii) a final refinement is obtained using a full Newton–Raphson minimization with a convergence criterium on the gradient norm of 10^{-6} or less.

3. RESULTS AND DISCUSSION

pMNEI was studied by means of ^{1}H 2D experiments at 400 and 500 MHz in aqueous solution at low pH (2.8). Non-sequential assignment was performed by a combination of DQF-COSY, TOCSY and NOESY spectra in H_2O and in D_2O solutions. Fig. 1 shows a partial NOESY spectrum of pMNEI in H_2O.

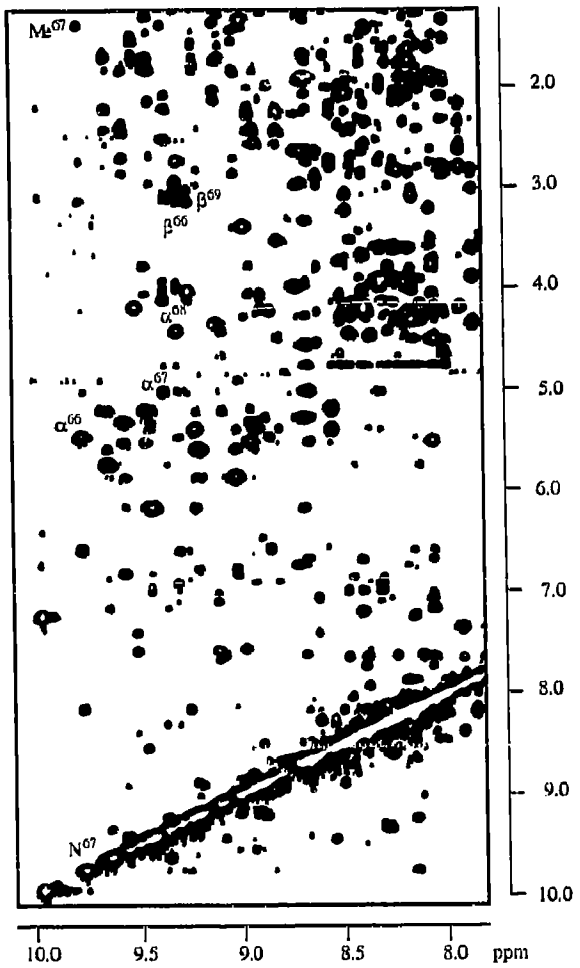

Fig. 1. Partial 500 MHz ^{1}H NOESY spectrum of pMNEI in H_2O.

The sequential assignment of pMNEI was performed by means of the MCD method, which allows the determination of the secondary structure of selected sequential segments without the need of the preliminary global assignment of all resonances to residue types. A likely active site of this very sweet protein is the loop connecting the β-strands from residue 59 to residue 78. It has been shown [13] that the corresponding peptide of the A chain of native monellin (Tyr[14]-Ala[15]-Ser[16]-Asp[17]) cross-reacts immunologically with an exposed segment of thaumatin (Tyr[57]-Phe[58]-Asp[59]) and that antibodies raised against monellin compete for aspartame. The loop Tyr[66]-Ala[67]-Ser[68]-Asp[69] contains residues that characterise all sweet peptides, i.e. an aromatic residue (Tyr[66]) and an acidic residue (Asp[69]), corresponding to F and D of aspartame, respectively.

Identification of the spin system of Ala[67] was sufficient, together with the recognition of the patterns of d_{NN}, $d_{\alpha N}$ and $d_{\alpha\alpha}$ typical of β-strands, to assign the whole segment 59–78. The detailed three-dimensional struc-

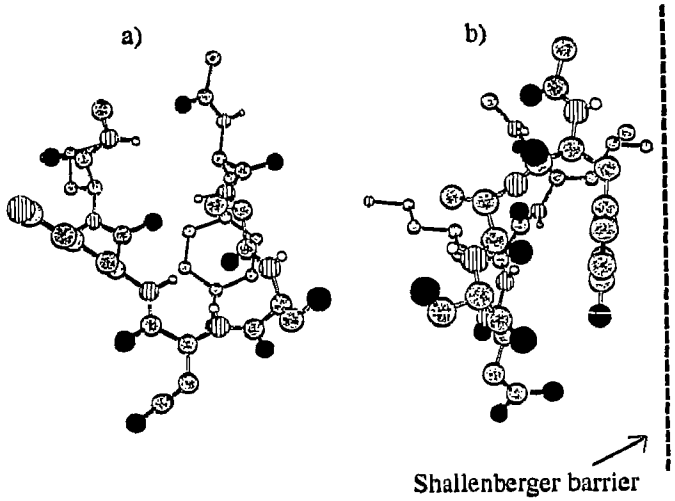

Fig. 2. Molecular model of Ac-Tyr-Ala-Ser-Asp-Lys-Leu-$NHCH_3$, determined from energy minimization. Panel a shows the view parallel to the main plane of the active site of the receptor; panel b shows the view at 90°, with the indication of the 'Shallenberger barrier', corresponding to the bottom of the active site of the receptor.

ture of the loop was determined on a model peptide, Ac-Tyr-Ala-Ser-Asp-Lys-Leu-$NHCH_3$, using several interresidue and intraresidue NOEs and subsequent energy minimisation. Such a procedure is justified, in our case, by the circumstance that the loop of native monellin protrudes from the bulk of the β-sheet, resulting in negligible side chain–side chain interactions with flanking residues. The number and relative intensities of NH–NH interactions suggest a type I β-turn as the characterising conformation of the loop. Starting from this simple structural indication and from the need of arranging the preceding and following residues in a regular β- strand, we built a three-dimensional model of the peptide and used it as a starting conformation for molecular mechanics (MM) calculations. In a first step we imposed, as the only constraint, the formation of an interstrand hydrogen bond involving the two blocking groups. The resulting model showed only minor violations of the observed NOEs; subsequent introduction of 20 NOE constraints led to a refined model consistent with all experimental data.

Fig. 2 shows the molecular model of the loop. It is very interesting to note that, thanks to the conformation of the loop, the side chains of the two key residues Tyr^{66} and Asp^{69}, corresponding to Phe and Asp of aspartame, are completely exposed: that of Tyr^{66} below the plane of the β-strand and that of Asp^{69} at the edge of the loop, so that they can easily interact with a receptor-site cavity open to the environment. The side chain conformation of Tyr^{66} is completely blocked by several interstrand interactions; that of Asp^{69} was optimised by a full search. The side view of the molecular model (Fig. 2b) shows that the ring of Tyr^{66} can be placed parallel to the so-called Shallenberger barrier (the bottom of the active site) while keeping the carboxyl of Asp^{69} at favourable distance from the B entity of the active site.

Fig. 3 shows the fit of these two side chains in our receptor model, together with the corresponding fit of aspartame. It is easy to see that the orientation of the side chains of Tyr^{66} and Asp^{69} corresponds closely to that of the proposed bioactive conformation of aspartame [1–3].

The role of the side chains of the residues flanking

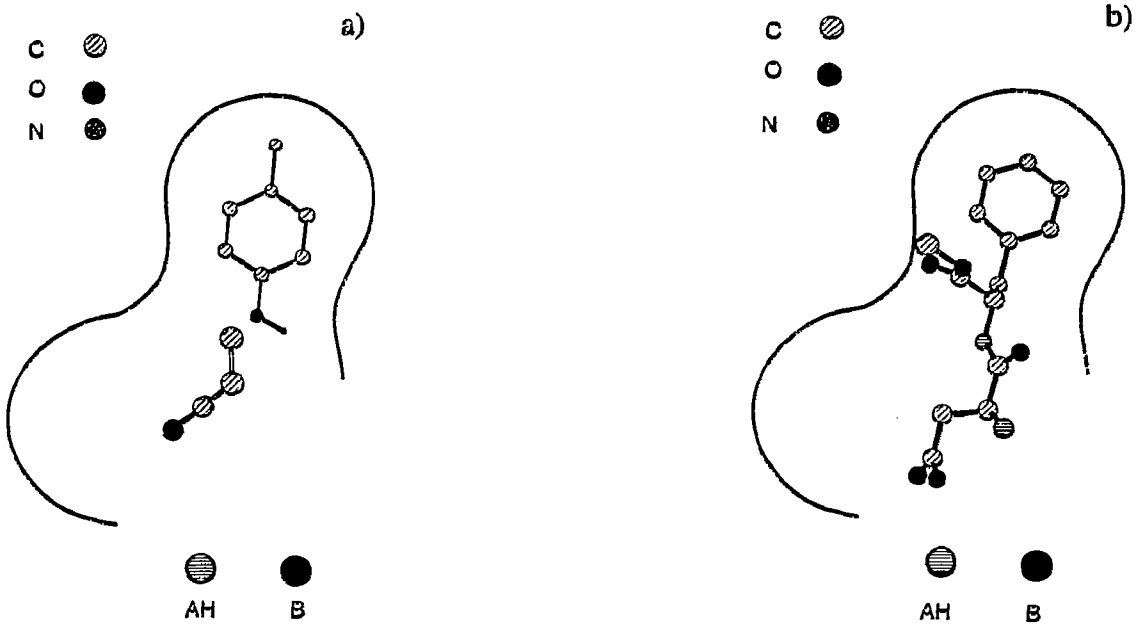

Fig. 3. Bidimensional contour of the active site of the sweet receptor. Panel a shows the fit of the side chains of Tyr^{66} and Asp^{69} of pMNEI; panel b shows the fit of the F_ID_{II} conformation of aspartame, previously proposed as the bioactive conformation of aspartame [1,3].

Tyr[66] and Asp[69] cannot be extrapolated as easily from known low molecular weight sweeteners but it is very likely that they contribute to the high molar activity of the protein and may lead to a more detailed mapping of the active site of the receptor.

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